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**Soybean extracts increase cell surface ZIP4 abundance and cellular zinc levels: a potential novel strategy to enhance zinc absorption by ZIP4-targeting**

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Short title: *Soybean extracts increase cell surface ZIP4 abundance*

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## ABSTRACT

Dietary zinc deficiency puts human health at risk. Therefore, we are exploring strategies for enhancing zinc absorption. In the small intestine, the zinc transporter ZIP4 functions as an essential component for zinc absorption. Overexpression of ZIP4 protein increases zinc uptake and thereby cellular zinc levels, suggesting that food components with the ability to increase ZIP4 could potentially enhance zinc absorption by the intestine. In this study, we used mouse Hepa cells, which regulate mouse Zip4 (mZip4) in a manner indistinguishable from that in intestinal enterocytes, to screen for suitable food components able to increase the abundance of ZIP4. Using this ZIP4-targeting strategy, two such soybean extracts were identified that were specifically able to decrease mZip4 endocytosis in response to zinc. These soybean extracts also effectively increased the abundance of apically localized mZip4 in transfected polarized Caco2 and Madin–Darby canine kidney cells and, moreover, apically localized two mZip4 acrodermatitis enteropathica mutants. Soybean components were purified from one extract and soyasaponin Bb was identified as an active component that increased both mZip4 protein abundance and zinc levels in Hepa cells. Finally, we confirmed that soyasaponin Bb is capable of enhancing cell surface endogenous human ZIP4 in human cells. Our results suggest that ZIP4 targeting may represent a new strategy to improve zinc absorption in humans.

## Summary statement

Zinc deficiency compromises human health. Here, we established a ZIP4-targeting strategy to identify potential enhancers of zinc absorption from dietary components. We found that soybean extracts, including soyasaponin Bb, can increase cell surface ZIP4 abundance and cellular zinc levels.

**Key words:** apical membrane, degradation, endocytosis, screening, soyasaponin Bb, zinc transporter.

## ABBREVIATIONS FOOTNOTE

AE, acrodermatitis enteropathica; CTB, cholera toxin subunit B; HRP, horseradish peroxidase; hZIP4, human ZIP4; MDCK, Madin–Darby canine kidney; mZip4, mouse Zip4; MT1, metallothionein 1; RT-qPCR, Reverse-transcription quantitative polymerase chain reaction; SEAP, secretory alkaline phosphatase; SHG, soyaflavone HG; SSA, soyhealth SA; Tf, transferrin; TfR, transferrin receptor; TPEN, N,N,N9,N9-tetrakis (2-pyridylmethyl) ethylenediamine.

## INTRODUCTION

Zinc is an essential nutrient for human health and is an important structural, catalytic, and regulatory component of proteins in a diverse range of physiological processes [1-3]. Thus, severe zinc deficiency causes a broad range of defects including diarrhea, alopecia, skin lesions, immune system dysfunction, taste dysfunction, growth retardation, hypogonadism, and neurological disorders [4-6]. Zinc deficiency is a serious nutritional problem in developing countries [7-9], and recent studies have reported individuals with marginal zinc deficiencies and a suboptimal zinc status in both less developed and industrialized countries [5, 10]. In particular, elderly individuals have an increased likelihood of developing a zinc deficiency, as documented by a decline of serum or plasma zinc levels with age [11-13], leading to an increased risk of developing chronic diseases [14]. In these cases, restoration of zinc levels has improved biological activities such as immune functions and stress response systems [12, 15, 16].

Common causative factors of marginal zinc deficiency include foods rich in inhibitors of zinc absorption and/or containing small amounts of bioavailable zinc [17, 18]. Although the efficiency of dietary zinc absorption approaches 90% during severe restriction, it is estimated to be about 30% in normal conditions [19, 20]. Thus, increasing zinc absorption is important to prevent marginal zinc deficiency.

Little was known about the molecular mechanism of dietary zinc absorption in the intestine, but in 2002, mutations in the *SLC39A4* (*ZIP4*) gene were shown to result in the rare autosomal recessive genetic disorder acrodermatitis enteropathica (AE) [21, 22], which causes severe zinc deficiency [23-25]. The *ZIP4* protein functions as an essential zinc transporter for dietary zinc absorption in mammals, and plays pivotal roles in intestinal integrity [26, 27]. A number of studies including those of transfected cells in culture have revealed the molecular basis of *ZIP4* expression and functions [28-37]. Expression of *ZIP4* is dynamically regulated by multiple post-transcriptional mechanisms; during zinc deficiency, *ZIP4* mRNA is stabilized, and *ZIP4* protein accumulates on apical membranes by escaping from endocytosis and degradation [33]. It then undergoes processing by removal of the long extracellular amino-terminal half via proteolytic cleavage during prolonged zinc deficiency [34]. Overexpression of *ZIP4* increases cellular zinc levels [28, 29, 31, 32, 34, 38], suggesting that a strategy to increase *ZIP4* expression would be helpful in preventing zinc deficiency.

To increase dietary zinc absorption, a variety of strategies have been extensively explored [39]. Zinc-fortified flour is one such example that is a safe and appropriate strategy for enhancing zinc status [40]. Rice biologically fortified with zinc was also shown to increase zinc absorption [41]. Alternatively, increasing zinc bioavailability in food by



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reducing inhibitors of zinc absorption is possible [18, 20]. Because an inhibitory role of phytic acid on zinc absorption has been identified [17], reducing phytic acid in staple cereals is a promising means of enhancing zinc absorption [42]. By contrast, some dietary factors are shown to positively influence zinc absorption and bioavailability [17, 18, 20, 43, 44], but such dietary components need to be investigated further.

In this study, we established a unique screening method of dietary components that would enable the increase of both cell surface ZIP4 expression and cellular zinc levels. This technique identified soybean extracts as containing such components, which included soyasaponin Bb. Soyasaponin Bb increased the cell surface expression of both mouse Zip4 (mZip4) and human ZIP4 (hZIP4), as well as mutant mZip4 containing an AE-causing mutation. Because zinc absorption in the elderly is lower than in young adults following either a zinc-adequate or zinc-restricted diet [45], dietary components such as soyasaponin Bb have the potential to improve zinc nutrition in these vulnerable groups.

## EXPERIMENTAL

### Cell culture and stable transfection

Mouse Hepa cells, HeLa cells, and Madin–Darby canine kidney (MDCK) FLp-In™ T-Rex cells (a gift from Dr. Jack Kaplan, University of Illinois College of Medicine, Chicago, IL) [46] were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Multiser, Trace Scientific, Melbourne, Australia), 100 units penicillin/ml, and 100 µg streptomycin/ml. Caco2 cells stably expressing mZip4 (pools of stable clones, consisting of those expressing (20–30%) and not expressing mZip4 (70–80%) as previously described [34]) were maintained in the same conditions, except that FBS (Filtron Pty Ltd., Brooklyn, Australia) was used to optimize cell culture conditions. The cells were cultured for at least 12 days on 24-mm polyester membrane Transwell® plates with 0.4-µm pores (Greiner, Frickenhausen, Germany) to allow the formation of tight junctions. Human AsPC1 cells were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% FBS (Multiser, Trace Scientific), 100 units penicillin/ml, and 100 µg streptomycin/ml. Proteasome inhibitor MG132 (Peptide Institute Inc., Osaka, Japan) or lysosome inhibitor bafilomycin A1 (Sigma) was used to block protein degradation at the indicated final concentrations.

To monitor zinc levels in Hepa cells, which express no alkaline phosphatase activity in the spent medium, the secretory alkaline phosphatase (SEAP) reporter plasmid driven by the mouse *metallothionein 1* (*Mt1*) promoter (MT-SEAP reporter plasmid) was introduced. The

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MT-SEAP plasmid was constructed by ligating a fragment of the *MtI* promoter with a fragment of *SEAP* cDNA (Clontech, Palo Alto, CA). The *MtI* promoter contains five metal response elements and so can drive SEAP activity to reveal relative cellular zinc levels [47]. Hepa cells were transfected with this plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After transfection, clones stably harboring the plasmid were selected in medium containing 10 µg/ml puromycin (Sigma) for 2 weeks. To transiently express mZip4 or hZIP4 in HeLa cells, pcDNA3 containing either cDNA was transfected in the same manner. To establish MDCK FLP-In™ T-Rex cells stably expressing mZip4 carboxyl-terminally tagged with HA (mZip4-HA) and mZip4-HA carrying an AE-causing mutation (specifically P200L and L382P) [29, 34], the pcDNA™5/FTR/TO plasmid (Invitrogen) containing each cDNA was introduced using Lipofectamine 2000. Stable clones were selected in medium containing 400–500 µg/ml Hygromycin B (Nacalai Tesque) for 3 weeks. To generate zinc-deficient culture media, FBS was treated with Chelex-100 resin (Bio-Rad, Hercules, CA), as described previously [34]. Alternatively, 5 µM N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN; Dojindo Molecular Technologies Inc., Kumamoto, Japan) was added to the cell culture medium to chelate cytosolic zinc.

### **Screening of food components**

Most foodstuffs and products were purchased in the market (Kyoto, Japan). Some were donated by the Central Miso Research Institute (Tokyo, Japan), Yamada Bee Farm Grant for Honeybee Research (Okayama Japan), and the Food Science Institute Foundation (Kanagawa, Japan). Soyaflavone HG (SHG) and Soyhealth SA (SSA) were donated by Fuji Foundation for Protein Research and purchased from Fuji Oil (Osaka, Japan). Some extracts including SHG and SSA were prepared as an aqueous solution, and others were dissolved in methanol (MeOH), ethanol, dimethylsulfoxide, or ethyl acetate. Subconfluent Hepa cells or Hepa cells harboring MT-SEAP were cultured in medium containing 10% FBS supplemented with one of these extracts for 24–48 h. Total cellular proteins were prepared using alkaline phosphatase lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl<sub>2</sub>, and 0.1% Triton X-100) [48].

### **Generation of the anti-mZip4 monoclonal antibody**

Fused proteins consisting of the extracellular amino-terminal portion of mZip4 (332 amino acid residues from Met<sup>1</sup> to Ser<sup>332</sup>) and maltose binding protein were used as mZip4 antigens. The anti-mZip4 monoclonal antibody was produced as described previously [49]. An ascites was generated by the injection of  $1 \times 10^7$  hybridoma cells into pristine-primed mice.

### Immunoblot and lectin blot analyses

Total cellular protein (20 µg) or membrane protein (20 µg) was lysed in 6 × sodium dodecyl sulfate (SDS) sample buffer at 37°C for 30 min, separated by electrophoresis through 7.5% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Pall Corporation, East Hills, NY). The blot was blocked with blocking solution (1% skim milk and 0.1% Tween 20 in phosphate-buffered saline (PBS) or SuperBlock blocking buffer (Thermo Fisher Scientific, Rockford, IL) supplemented with 0.1% Tween 20 and then incubated with anti-mZip4 monoclonal antibody (1:1,000 dilution), anti-ZIP4 peptide polyclonal antiserum (1:600) that detects the cytoplasmic loop between transmembrane III and IV of both hZIP4 and mZip4 [33], anti-tubulin (Sigma, 1:20,000), anti-GRP78 (ABR, Golden, CO, 1:1,000), anti-transferrin receptor (Invitrogen, 1:1,000), anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:2,000), or anti-LC-3 (Cell Signaling, Danvers, MA, 1:2,000). Horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibodies (GE Healthcare, Waukesha, WI) was used at a 1:3,000 dilution for detection. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrates (Millipore, Billerica, MA) or Chemi-Lumi One L (Nacalai Tesque). Lectin blotting was performed using HRP-ConA (J-Oil Mills Inc., Tokyo, Japan) after blocking with blocking solution (0.5 M NaCl and 0.05% Tween 20 in 10 mM Tris-HCl, pH 7.4). The fluoroimage was obtained using a LAS1000 plus image analyzer (Fujifilm, Tokyo, Japan). Densitometric quantification of the band intensity was performed using ImageQuant TL software (GE Healthcare).

### Cell surface biotinylation and detection of mZip4

Hepa or AsPC1 cells and Caco2 or MDCK cells cultured on transwell plates were treated with or without soybean components for the indicated period. Before biotinylation, cells were washed twice with ice-cold PBS, and then EZ-Link, a Sulfo-NHS-SS-Biotin reagent (Pierce, Rockford, IL) was added to biotinylated lysine residues exposed on the extracellular surface. Biotinylated proteins were recovered from streptavidin-coupled beads in 6 × SDS sample buffer and then immunoblotted.

### SEAP assay

The culture supernatant was incubated at 65°C for 30 min to inactivate serum-derived phosphatases [50], then 10 µl of medium was used to measure SEAP activity. One hundred microliters of substrate solution (2 mg/ml *p*-nitrophenyl phosphate dissolved in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl<sub>2</sub>) was added, and *p*-nitrophenol

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released by SEAP was measured by the absorbance at 405 nm. In the cases of colored extracts or soybean components, the absorbance was calculated by subtracting the blank absorbance from the total absorbance. SEAP activity was calculated from the standard curve drawn using calf intestine alkaline phosphatase (Takara-Bio Inc., Otsu, Japan) as a standard. Total SEAP activity in the spent medium was normalized by total cellular protein.

### **Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was isolated from Hepa or MDCK cells using Sepasol I (Nacalai Tesque), according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan), and real-time PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo). Samples were denatured at 95°C for 2 min, and amplified for 40 reaction cycles involving denaturation at 95°C for 15 s, annealing at 56°C for 15 s, and extension at 72°C for 30 s per cycle. Specificity of the PCR reaction was confirmed by acrylamide electrophoresis. Each cDNA sample was prepared in triplicate, and the same reaction was performed without reverse transcriptase as a negative control. *β-actin* or *GAPDH* primers were used to normalize each sample. The amount of each mRNA was evaluated by threshold cycle (Ct) values, and relative levels of each mRNA were evaluated by the values of  $2^{[Ct(\beta\text{-actin or GAPDH}) - Ct(\text{each mRNA})]}$ . Primer sequences are described in the supplementary material.

### **MTT assay**

MTT assays were performed as previously described [51]. Briefly, Hepa cells were cultured on 96-well plates in the presence of SHG or SSA for 24 h, then were incubated with 0.5 mg/ml 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Dojindo Laboratories, Kumamoto, Japan) for 4 h. MTT was reduced to blue formazan crystals during this incubation. Cells were lysed into 100 µl of lysis solution (10% SDS, 10 mM NH<sub>4</sub>Cl) for 16 h. Absorbance was measured at 595 nm.

### **Fluorescent microscopy**

Hepa cells cultured on coverslips were treated with or without SHG and SSA for 36 h and then incubated with 1 µg/ml Alexa Fluor 488-conjugated cholera toxin subunit B (CTB; Invitrogen) or 10 µg/ml Texas Red-conjugated transferrin (Tf; Rockland, Gilbertsville, PA) for an additional 0.5 or 1 h. Cells were washed twice with PBS on ice and fixed with 4% paraformaldehyde. After washing, the coverslips were mounted using antifade mounting medium (Invitrogen), and were observed under a fluorescent microscope (Olympus, Tokyo, Japan).

## Statistical analyses

All data are depicted as means  $\pm$  SD. Statistical significance was determined by the Student's *t*-test and accepted at  $P < 0.05$ .

## RESULTS

### Screening system for food components with the ability to increase mZip4 expression

We previously reported that mouse Hepa cells mimic the dynamic modulation of mZip4 expression in the small intestine of mice in multiple post-transcriptional mechanisms at both the mRNA and protein levels [33, 34], but that the localization of mZip4 to the cell surface of Hepa cells had not been examined. We generated an anti-mZip4 monoclonal antibody (Figure S1A), and examined the localization of mZip4 in Hepa cells using a surface biotinylation assay with a membrane-impermeable biotinylation reagent. The biotinylated mZip4 protein was detected in cells cultured in zinc-deficient medium (Figure S1B), indicating that mZip4 is localized to the cell surface in Hepa cells during zinc-deficient conditions, as in intestinal epithelial cells. These characteristics of Hepa cells appear to be useful in screening food components that directly affect mZip4 accumulation at the cell surface.

To efficiently perform the screening of such food components, we stably transfected the MT-SEAP plasmid into Hepa cells to monitor cellular zinc levels (Figure S1C). Because the *MtI* promoter has five metal response elements, its activity is zinc-dependent so it can act as a physiologically relevant readout of increased intracellular zinc. The addition of the zinc chelator TPEN into the culture medium of Hepa cells harboring MT-SEAP increased mZip4 expression but decreased SEAP activity (Figure S1D), indicating that the measurement of SEAP activity could discriminate false-positive food components directly affecting mZip4 expression through decreasing cellular zinc levels.

### SHG and SSA increase mZip4 expression and cellular zinc levels

There are no reports about foodstuffs that modulate ZIP4 expression. We screened extracts prepared from foodstuffs and products such as beans, cereals, meats, egg, milk, and honey using Hepa cells, and found a number of extracts to enhance mZip4 expression. In particular, extracts derived from fermented soybean paste or soybean products highly enhanced mZip4 expression. Among these, two commercial soybean extracts, SHG and SSA, enhanced mZip4 expression in a dose-dependent manner (Figure 1A), which paralleled the increased SEAP activity in spent medium (Figure 1B). Endogenous *MtI* mRNA expression was increased 6 h after treatment with SHG and SSA (Figure 1C), at which time mZip4 expression was also



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increased (Figure 1D), indicating that SHG and SSA increase cellular zinc levels. Importantly, longer treatment with SHG and SSA did not cause severe toxicity in Hepa cells, although higher concentrations of SHG slightly retarded cell growth (Figure 1E). Thus, SHG and SSA appear to have the unique ability to improve zinc absorption.

### **SHG and SSA decrease endocytosis and the degradation of mZip4 protein**

Cell surface mZip4 expression is enhanced in zinc-deficient conditions through both increased mRNA stability and decreased endocytosis from the cell surface, resulting in less degradation [28, 33, 34]. In Hepa cells, neither SHG nor SSA treatment increased mZip4 mRNA expression (Figure 2A), but SHG and SSA treatment blocked mZip4 degradation triggered by the addition of zinc (Figure 2B): mZip4 immunoreactivity, which was increased in Hepa cells cultured in zinc-deficient conditions for 40 h, was decreased by a 4 h culture with ZnSO<sub>4</sub> (Figure 2B, lane 3), and this was blocked by SHG and SSA (Figure 2B, lanes 4 and 5). Hence, it appeared that SHG and SSA inhibited the endocytosis and/or degradation of mZip4 protein.

We next examined whether SHG and SSA could block mZip4 protein degradation by inhibiting the mZip4 cellular degradation pathway. In Hepa cells, endocytosed mZip4 was mainly degraded via the ubiquitin–proteasome degradation pathway (Figure 2C), although a minor contribution was made by the lysosomal pathway, because MG132 treatment blocked the rapid loss of mZip4 immunoreactivity triggered by zinc replenishment to a greater extent than bafilomycin A1 treatment (Figure 2C). We therefore examined the possibility that SHG and SSA affected the functions of the proteasomal degradation pathway, but found that neither significantly altered the amounts of ubiquitinated proteins in the presence or absence of MG132 in Hepa cells (Figure 2D). Moreover, MG132 did not significantly affect the increase in mZip4 expression that occurred following SHG and SSA treatment (Figure 2D). In a surface biotinylation assay, biotinylated mZip4 was clearly detected in lysates prepared from Hepa cells cultured in medium containing SHG and SSA for 36 h (Figure 2E). These results indicate that SHG and SSA increase cell surface mZip4 expression by decreasing its endocytosis and degradation.

To exclude the possibility that the reduction of mZip4 endocytosis by SHG and SSA could be attributed to their broad inhibition of overall endocytosis, we examined the endocytosis of cell surface transferrin receptor (TfR), which is constitutively endocytosed via clathrin-coated vesicles [52]. Expression levels of biotinylated TfR showed almost no change after SHG and SSA treatment, suggesting that neither block TfR endocytosis (Figure 2E). This was confirmed by the fact that the endocytosis of Texas Red-conjugated Tf, which binds and endocytoses with surface TfR, was not impaired by SHG or SSA treatment (Figure 2F,

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upper panels). Moreover, the alternative endocytic pathway via lipid rafts was not impaired by SHG or SSA treatment, because CTB conjugated with Alexa Fluor 488, which enters the cells by binding to the lipid raft ganglioside GM1 [53] and traveling to the Golgi/endoplasmic reticulum [54], typically accumulated at the perinuclear region corresponding to the Golgi apparatus (Figure 2F, lower panels). Intracellular signals of both Texas Red and Alexa Fluor 488 were not detected at 0 min even at 37°C (lane 2) or at 4°C (lane 3), excluding the possibility of nonspecific binding. Taken together, SHG and SSA appear to cause an increase in mZip4 expression at the plasma membrane by specifically decreasing the endocytosis of mZip4.

### **SHG and SSA increase exogenously expressed mZip4 accumulation at the apical membrane of polarized Caco2 cells**

Many studies assessing the effects of food components on nutrient absorption have been carried out using Caco2 cells, which have similar morphological and functional characteristics to absorptive small intestinal cells [39, 55, 56]. Moreover, important information regarding zinc absorption has been reported from Caco2 cell assays [41, 43, 57, 58]. However, we could not detect hZIP4 expression in CaCo2 cells by immunoblot analysis, even when cells were cultured in zinc-deficient conditions (see below). Therefore, we examined the effects of SHG and SSA on mZip4 expression in CaCo2 cells stably transfected with mZip4 carboxyl-terminally tagged with an HA epitope, in which expression was driven by the CMV promoter [34]. CaCo2 cells were cultured in transwell plates for 12 days until polarized, and were then treated with SHG and SSA on the apical side for 24 or 48 h. Surface biotinylation reagent was applied from the apical compartment of the transwell plate, and the presence of biotinylated mZip4 protein was examined (Figure 3). SHG and SSA increased mZip4 protein exogenously expressed on the apical surface of polarized CaCo2 cells (*biotinylation* in Figure 3A, *right* panels in Figure 3B) as well as in total cell lysates (*input* in Figure 3A, *left* panels in Figure 3B). In these experiments, the expression of mZip4 protein, corresponding to full-length mZip4 (~75 kDa), was increased by SHG and SSA, but that of the processed mZip4 peptide (the carboxyl terminal peptide of mZip4 of ~37 kDa) was not. Full-length mZip4 was not significantly increased in zinc-deficient conditions (CX lane in Figure 3A, *right* panel), while the processed mZip4 peptide was increased on the cell surface, as previously described [34]. These results confirm that the ability of SHG and SSA to increase mZip4 expression is not cell-type specific.

### **SHG and SSA increase AE-causing mZip4 mutant accumulation at the apical membrane**



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To investigate whether SHG and SSA directly affect apically localized mZip4 expression in more detail, we established MDCK (MDCK FLp-In™ T-Rex) cells stably expressing mZip4 carboxyl-terminally tagged with an HA epitope. Expression of mZip4 was under control of a Tet-regulatable promoter in MDCK cells, so was only activated when the cells were treated with doxycycline (Dox) (Figure 4A, lanes 1 and 2). After a further culture in normal medium for 24 h, the cells were cultured in normal medium in the presence or absence of SHG or SSA for up to 48 h (Figure 4A, lanes 3–9). In contrast to the gradual decrease of mZip4 expression in cells cultured in the absence of SHG or SSA (Figure 4A, lanes 3–5), mZip4 expression levels were almost unchanged in cells cultured with SHG or SSA (Figure 4A, lanes 6–9). Using the polarized MDCK cells, we showed that biotinylated mZip4 protein levels on the apical surface increased in the presence of SHG and SSA for 48 h (Figure 4B). To investigate whether this apically accumulated mZip4 protein could increase cellular zinc levels, we measured *Mt1* mRNA expression in MDCK cells treated with SHG and SSA after Dox treatment, and compared expression levels with those of control cells. *Mt1* mRNA expression was significantly enhanced by Dox treatment for 24 h (Figure 4C, *left*), and this was decreased after removal of Dox. SHG and SSA increased *Mt1* expression by 4.0-fold or 2.7-fold at 24 h, and 4.4-fold or 3.2-fold at 48 h, respectively, compared with *Mt1* expression in their absence (Figure 4C, *right*). Importantly, this was observed following the addition of SHG and SSA to the cell culture medium from the apical compartment of the transwell plate. Taken together, SHG and SSA possess the ability to increase mZip4 protein expression on the cell surface, thereby increasing cellular zinc levels.

Next, we examined the effect of SHG and SSA on the expression of mZip4 mutants corresponding to those found in AE patients using MDCK FLp-In™ T-Rex cells. Specifically, we chose P200L and L382P mutants because both were shown to locate to the cell surface [29, 34]. Both P200L and L382P mutants expressed in MDCK cells were detected by two bands, the upper corresponding to the highly glycosylated form localized to the plasma membrane [29]. We confirmed that SHG and SSA increased the expression of the upper bands of both AE mutants of mZip4 (Figure 4D). Moreover, a cell surface biotinylation assay indicated that SHG and SSA increased the apical accumulation of the mZip4 P200L mutant (Figure 4E). These results indicate that SHG and SSA both increase mZip4 expression, even in the case of some AE mutants.

## **Soyasaponin Bb is an active component that increases surface mZip4 expression**

SSA contains large quantities of soyasaponins, while SHG contains both isoflavones (~50%) and soyasaponins [59]. We therefore hypothesized that soyasaponins may be the component responsible for increasing surface mZip4 abundance. Based on this assumption, we purified

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one active fraction containing an active compound, which was confirmed as a single peak on reversed-phase high-performance liquid chromatography (HPLC) (Figure S2A). Its chemical structure was determined to be soyasaponin Bb by spectral data including nuclear magnetic resonance analyses (Figure S2B) (see supplementary material) [60]. Soyasaponin Bb increased mZip4 expression and *Mt1* mRNA expression to levels similar to those seen following SSA treatment, but at a 10-fold lower concentration (Figure 5A and B), without increases of mZip4 mRNA expression (Figure 5C). This was in agreement with its content ( $14.4 \pm 0.34\%$  (w/w) ( $n = 3$ )) in SSA, which we calculated from the standard curve based on the HPLC peak area of the absorbance at 210 nm. SHG contains less soyasaponin Bb ( $0.42 \pm 0.01\%$  (w/w) ( $n = 3$ )), and was also in agreement with its effect on mZip4 expression. This suggests that soyasaponin Bb may also be an active component of SHG, and this is currently under investigation. Additionally, soyasaponin Bb increased expression of AE-causing mZip4 mutants (Figure 4D and E), which indicates its efficacy at enhancing mZip4 expression.

### **Soyasaponin Bb increases cell surface hZIP4 expression**

Finally, we examined whether soyasaponin Bb could enhance the surface expression of hZIP4. Recent studies indicated that the human pancreatic carcinoma cell type AsPC1 expresses hZIP4 [38, 61], although its surface expression and zinc-responsive expression have not yet been closely examined. We confirmed that hZIP4 expression and its cell surface localization increased in AsPC1 cells during zinc-deficient culture (Figure 6A and B). Because we were unable to detect hZIP4 expression in Caco2 cells (Figure 6A), as described earlier, we evaluated the effect of soyasaponin Bb on hZIP4 expression using AsPC1 cells. Soyasaponin Bb enhanced hZIP4 expression (Figure 6C) and led to the surface accumulation of hZIP4 protein (Figure 6D) in AsPC1 cells. Moreover, as seen in Hepa cells, soyasaponin Bb blocked hZIP4 degradation triggered by the addition of zinc in AsPC1 cells: hZIP4 immunoreactivity, which was increased in AsPC1 cells cultured in zinc-deficient conditions for 24 h, was decreased by an 8 h culture with  $\text{ZnSO}_4$ , and this was blocked in medium containing soyasaponin Bb (Figure 6E). These results suggest that soyasaponin Bb increases cell surface hZIP4 expression by decreasing its degradation, suggesting that it is a potentially useful dietary component for improving zinc absorption.

## **DISCUSSION**

Iron and copper are converted to their reduced forms prior to uptake by intestinal epithelial cells, but zinc does not undergo such a redox reaction during an absorption process. This

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feature of zinc suggests that the expression levels of ZIP4 at the apical membrane of intestinal epithelial cells are of critical importance for defining net zinc absorption. Therefore, the ZIP4-targeting strategy conducted in this study has the potential to make efficient zinc absorption possible. We identified soybean extracts such as SHG and SSA as having the ability to enhance mZip4 expression, and, moreover, identified soyasaponin Bb as an active enhancer in SSA. The efficacy of SHG, SSA, and soyasaponin Bb on increasing ZIP4 expression was also confirmed by mZip4 mutants involved in AE pathogenesis, as well as hZIP4. To our knowledge, this is the first report of a food component modulating cell surface ZIP4 abundance.

In this study, we focused on ZIP4-targeting, so the characteristics of Hepa cells, which express high levels of endogenous mZip4 protein in a zinc-dependent manner, are particularly valuable. We reinforced the values of Hepa cells by expressing MT-SEAP, which enabled us to determine whether food components actually increased mZip4 expression or only appeared to do so by chelating cellular zinc (see Figure S1D). In addition to the zinc-dependence of *Mt1* promoter activity, SEAP activity also depends on cellular zinc levels [50, 62]. These features are beneficial in exploring food components that directly affect mZip4 expression in Hepa cells, because they decrease the rate of obtaining false positives.

We showed that SHG and SSA did not significantly affect clathrin- and caveolae-dependent endocytosis pathways by the use of Tf/TfR and CTB proteins, which are both thought to operate in the endocytosis of ZIP4 [31, 32, 34]. This suggested that SHG and SSA contain compound(s) that specifically inhibit mZip4 endocytosis by direct interaction. Subsequently, we identified the amphiphilic triterpene glycoside soyasaponin Bb as the active compound from SSA. Soyasaponin Bb enhances mZip4 expression, while its aglycone part soyasapogenol B (a membrane permeable compound) was unable to enhance mZip4 expression<sup>1</sup>. If soyasaponin Bb directly interacts with mZip4, it may do so at the extracellular side because ZIP4 has a long extracellular amino-terminal region. This hypothesis is supported by our finding that SHG and SSA did not increase the expression of processed mZip4 (mZip4 lacking the long amino-terminal extracellular region) (see Figure 3A), which was shown to be localized to the plasma membrane [34]. We found that several extracts prepared from other foods also increased mZip4 expression<sup>2</sup>, so identification of the active component(s) from these extracts may be useful to clarify this point.

Soybeans contain moderate levels of zinc but are not regarded as a good source of absorbable zinc because they have large amounts of phytate, a potent inhibitor of zinc absorption [63, 64]. However, a study on zinc nutritional status in Japan revealed a positive correlation between zinc intake from soybean foods and serum zinc levels [13]. This may be attributed to the Japanese dietary habit in which soybeans are fermented to processed foods

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such as miso (fermented soybean paste), which shows reduced phytate contents [65]. In the present study, we found that some types of miso can increase mZip4 expression<sup>3</sup>, and therefore soyasaponin Bb may synergistically support zinc absorption in the fermented Japanese diet. The next critical step for our work will be to evaluate whether a diet including soyasaponin Bb contributes to the potential physiological consequences of zinc absorption *in vivo*.

Another important aspect of this study was our use of AsPC1 cells to verify that soyasaponin Bb enhances hZIP4 expression. Many studies assessing the effects of food components on nutrient absorption, including zinc, have been carried out using Caco2 cells, which have similar morphological and functional characteristics to absorptive small intestinal cells [39, 41, 43, 57, 58]. However, in the present study, we detected insufficiently high hZIP4 protein levels in Caco2 cells (see Figure 6A). By contrast, AsPC1 cells expressed high levels of hZIP4 [38, 61], which was zinc-responsive (see Figure 6A). Other cell types, such as Hepa and AsPC1 cells, express ZIP4 in a zinc-deficient dependent manner, so would be useful to investigate the molecular mechanism of ZIP4 regulation, and to examine the interaction of ZIP4 protein with food components from the viewpoint of ZIP4-targeting. However, it should be remembered that Hepa and AsPC1 cells are both carcinoma cell types and not polarized [38, 61, 66], although this may be less important for food components such as soyasaponin Bb, which modulate ZIP4 expression from the extracellular side.

In conclusion, we screened food components with the ability to increase mZip4 expression and cellular zinc levels in Hepa cells, and identified two soybean extracts: SHG and SSA. The effects of SHG and SSA could be attributed to both a decrease of mZip4 endocytosis from the cell surface/apical membrane and degradation of mZip4. We identified soyasaponin Bb as one of the active components of SSA, which is the first food component known to modulate cell surface ZIP4 abundance. The efficacy was confirmed by the accumulation of AE-causing mZip4 mutants and hZIP4 in the cells treated with them. Dietary zinc contents often fail to meet recommendations [67], which increases the risk of developing chronic disease, particularly in the elderly. Hence, food-based strategies enabling efficient zinc absorption from the diet are of particular importance. Our present novel results are a major step forward in preventing zinc deficiency and in improving human health.

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## DECLARATION OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

A.H., and T.K., study design; A.H., K.O., M.T., K.K., H.N., S.E., Y.M., S.M., M.N., K.I., H.O., G.A., and T.K., the collection, analysis, and interpretation of data; A.H., M.T., and T.K., the writing of the report; and A.H., M.T., and T.K., the decision to submit the manuscript for publication.

<sup>1</sup> Takahashi and Kambe, unpublished data

<sup>2</sup> Hashimoto and Kambe, unpublished data.

<sup>3</sup> Hashimoto and Kambe, unpublished data.



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## FIGURE LEGENDS

### Figure 1 SHG and SSA enhance expression of mZip4 and increase cellular zinc levels in Hepa cells.

(A) SHG and SSA dose-dependently increase mZip4 expression in Hepa cells. Total proteins (20  $\mu$ g) prepared from Hepa cells cultured in the presence of indicated concentrations of SHG or SSA for 24 h were analyzed by immunoblot analysis using an anti-mZip4 monoclonal antibody. (B) SHG and SSA dose-dependently increased SEAP activity in Hepa cells harboring MT-SEAP. Each value is the mean  $\pm$  SD of three independent experiments (\*\* $P < 0.01$  vs. conditions without SHG or SSA). Cells were cultured in the presence of indicated concentrations of SHG or SSA for 24 h. (C) SHG and SSA increase *Mt1* mRNA expression in Hepa cells. Total RNA prepared from Hepa cells cultured in normal medium (N), zinc-deficient medium (CX), and in the presence of 100  $\mu$ M ZnSO<sub>4</sub> (Zn), 1.0% SHG, or 0.1% SSA for 6 h was subjected to RT-qPCR. Changes in *Mt1* mRNA expression relative to that in normal medium are shown after normalization with  $\beta$ -actin mRNA expression. Values are means  $\pm$  SD ( $n = 3$ , \* $P < 0.05$ ). (D) Time course of increases of mZip4 expression by SHG and SSA treatment or zinc-deficient culture. Note that mZip4 expression increases after 6 h of treatment. Hepa cells were cultured in the same conditions as (C) for indicated times. Tubulin is shown as a loading control in (A) and (D). (E) SHG and SSA do not have severe toxicity. MTT assays were performed as described in the EXPERIMENTAL section.

### Figure 2 SHG and SSA inhibit mZip4 degradation and mZip4 endocytosis.

(A) SHG and SSA did not increase mZip4 mRNA expression in Hepa cells treated as in Figure 1C. (B) SHG and SSA blocked mZip4 degradation in Hepa cells. Hepa cells were cultured in zinc-deficient conditions for 40 h, then cultured in the presence or absence of 20  $\mu$ M ZnSO<sub>4</sub> (Zn) with 1.0% SHG or 0.1% SSA for 4 h. Total cell lysates were prepared and subjected to immunoblot analysis. Band intensities were quantified by densitometric analysis after normalizing the mZip4 band intensity to that of tubulin, and are shown relative to that of Hepa cells cultured in normal medium (N) below each lane. The panels show the representative results of triplicate experiments. (C) MG132 more significantly blocked the rapid loss of mZip4 immunoreactivity triggered by the replenishment of zinc to zinc-deficient media than bafilomycin A1 (BafA1). The indicated concentration of MG132 or bafilomycin A1 was added to zinc-deficient medium (CX) just before the addition of 4  $\mu$ M ZnSO<sub>4</sub>, and incubated for 4 h. Note that



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MG132 treatments increased ubiquitinated proteins, while bafilomycin A1 increased the intensity of the LC-3II band (lower band), which was converted from LC-3I (upper band) during the autophagic process. (D) SHG and SSA did not significantly affect the amount of ubiquitinated proteins. Hepa cells were cultured in normal medium (N), or in the presence of 1.0% SHG or 0.1% SSA for 24 h, and then treated with or without 20  $\mu$ M MG132 for 4 h. Note that mZip4 abundance increased by SHG and SSA was also not affected by MG132 treatment. MG132 was prepared in a DMSO solution. Tubulin was used as a loading control in (B, C, and D). (E) SHG and SSA enhanced the surface abundance of mZip4, but did not significantly affect TfR expression. Hepa cells cultured in normal medium (N), zinc-deficient medium (CX), or in the presence of 20  $\mu$ M ZnSO<sub>4</sub> (Zn), or 1.0% SHG, 0.1% SSA for 36 h were washed with PBS and then treated with the biotinylation reagent sulfo-NHS-SS-biotin, as described in the EXPERIMENTAL section. Solubilized proteins were captured using streptavidin beads and analyzed by immunoblot analysis using an anti-mZip4 monoclonal antibody. Input refers to aliquots of the biotinylated proteins before avidin capture (i.e., total cell lysate), while biotinylation refers to avidin-captured proteins. GRP78 and ConA (lectin blotting) were used as loading controls for input and the biotinylation, respectively. mZip4 band intensities were quantified by densitometric analysis after normalizing the mZip4 band intensity to that of GRP78 for the input (*left panel*) or to that of ConA for the biotinylation (*right panel*), and is shown relative to that of Hepa cells cultured in normal medium (N) below each lane. The panels show the representative results of triplicate experiments. (F) Endocytosis of Tf and CTB were not significantly blocked by SHG and SSA. Hepa cells cultured as in (E) were additionally treated with 10  $\mu$ g/ml Tf-conjugated with Texas Red for 1 h or 1  $\mu$ g/ml Alexa Fluor 488-conjugated CTB for 0.5 h. The results of the control experiment performed at 0 min or at 4°C treated with 20  $\mu$ g/ml Tf-conjugated with Texas Red for 1 h or 1  $\mu$ g/ml Alexa Fluor 488-conjugated CTB for 30 min are also shown (*left panels*).

**Figure 3 SHG and SSA increased mZip4 expression apically expressed in polarized Caco2 cells.**

(A) SHG and SSA increased the apical localization of mZip4 exogenously expressed in Caco2 cells. Caco2 cells stably expressing mZip4 carboxyl-terminally tagged with HA epitope were grown in transwell plates for 12 days until polarized and then cultured in



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medium in the presence or absence (N) of 1.0% SHG or 0.1% SSA, or in zinc-deficient medium (CX) for 24 h, and cell surface biotinylation assays were performed. The biotinylation reagent sulfo-NHS-SS-biotin was added to the apical compartment of the transwell plate. Solubilized proteins were captured using streptavidin beads and analyzed by immunoblot analysis using an anti-HA antibody. Input refers to aliquots of total biotinylated lysates before avidin capture. Tubulin and ConA are shown as loading controls. The panels show the representative results of triplicate experiments. \* indicates the position of the processed mZip4 peptide, which contains the carboxyl terminal transmembrane domains of mZip4 [34]. (B) The intensities of mZip4 bands (full-length mZip4 peptide) in Caco2 cells cultured as in (A) for 24 or 48 h were quantified by densitometric analysis as in Figure 2E. Each value is the mean  $\pm$  SD of triplicate experiments (\* $P$ <0.05, vs. normal medium (N)). *Left graph, input; right graph, biotinylation*

**Figure 4 SHG and SSA increase mZip4 expression apically expressed in polarized MDCK cells.** (A) SHG and SSA increased mZip4 protein exogenously expressed in MDCK cells. Schematic representation of the time course used in the study is shown on the left. MDCK cells stably expressing mZip4 carboxyl-terminally tagged with HA epitope were cultured until confluent, and treated with Dox for 24 h. The cells were incubated in normal medium (N) for 24 h after washing with PBS, then incubated in the presence or absence of 1.0% SHG or 0.1% SSA for the indicated periods (0–48 h). Total cell lysates (5  $\mu$ g) prepared from cells were immunoblotted using an anti-HA antibody. Tubulin is shown as a loading control. (B) SHG and SSA increased the apically localized mZip4 expression in MDCK cells. MDCK cells were grown in transwell plates until polarized and then cultured in medium containing Dox (+) for 24 h, followed by incubation in normal medium for 24 h as in (A). Cells were then treated with or without (N) 1.0% SHG or 0.1% SSA for 48 h, and cell surface biotinylation assays were performed as in Figure 3A. The ratio was quantified and is shown as described in Figure 2E. (C) SHG and SSA increased cellular zinc levels in MDCK cells. Total RNA, which was prepared from MDCK cells treated with or without (N) 1.0% SHG or 0.1% SSA for 24 or 48 h after Dox removal followed by 24 h culture in normal medium as in A, was subjected to RT-qPCR. Relative changes in *Mt1* mRNA expression are shown after normalization to  $\beta$ -actin mRNA expression. In the *left graph*,

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*Mt1* mRNA expression in cells cultured without (–) Dox is defined as 1. In the *right* graph, *Mt1* mRNA expression in cells cultured without extracts (N) is defined as 1 (values are means  $\pm$  SD;  $n = 3$ ,  $*P < 0.05$ ). (D) SHG and SSA increased the expression of mZip4 mutants corresponding to those found in AE patients. MDCK cells stably expressing WT or mutant mZip4 (P200L and L382P) carboxyl-terminally tagged with HA epitope were cultured with Dox for 24 h, then treated with or without (N) 1.0% SHG or 0.1% SSA or 0.01% soyasaponin Bb for 48 h after washing with PBS, and total cell lysates (10  $\mu$ g) prepared from the cells were immunoblotted as in (A). Note that SHG and SSA increased expressions of both mZip4 mutants as in WT. GRP78 is shown as a loading control. (E) SHG and SSA increased the apically localized P200L mZip4 mutant as in WT. Cell surface biotinylation assays were performed, and the ratio was quantified and is shown as described in B. In (A, B, D and E), the panels show the representative results of triplicate experiments. In (D and E), Bb; Soyasaponin Bb (see Figures 5 and 6).

### Figure 5 Soyasaponin Bb is an active compound with the ability to enhance mZip4 expression.

(A) Soyasaponin Bb (Bb) remarkably enhanced mZip4 expression. Note that the soyasaponin Bb concentration that increased mZip4 expression was almost one-tenth that of SSA. Hepa cells were cultured in the presence of indicated conditions for 24 h. MeOH; methanol. Soyasaponin Bb was prepared in a MeOH solution. (B, C) Soyasaponin Bb (Bb) increased *Mt1* mRNA expression (B) but did not increase *mZip4* mRNA expression (C). Hepa cells were cultured in normal medium (N) or in the presence of 0.1% SSA or 0.01% Bb. *Mt1* and *mZip4* expression was measured as described in Figures 1C and 2A.

### Figure 6 SHG and SSA increased hZIP4 expression.

(A) Comparison of ZIP4 protein expression levels between Hepa, Caco2, and AsPC1 cells (*left* panel). Membrane proteins (20  $\mu$ g) prepared from cells cultured in medium containing normal medium (N), medium supplemented with 20  $\mu$ M ZnSO<sub>4</sub> (Zn), or zinc-deficient medium (CX) for 24 h were immunoblotted using an anti-ZIP4 peptide polyclonal antiserum. The antiserum detects hZIP4 and mZip4 at almost the same efficiency (*right* panel). Total cell lysates (10  $\mu$ g) prepared from HeLa cells transiently transfected with mZip4 or hZIP4 expression plasmid were immunoblotted. Both hZIP4

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and mZip4 expressed were tagged with an HA epitope. Lane 1, non-transfected; lane 2, mZip4-HA transfected; lane 3, hZIP4-HA transfected. (B) AsPC1 cells cultured as in (A) were subjected to cell surface biotinylation assays as in Figure 2E. The ratio was quantified by densitometric analysis after normalizing the hZIP4 band intensity to that of tubulin for the input (*left* panel) or to that of ConA for the biotinylation (*right* panel), and is shown relative to that of AsPC1 cells cultured in normal medium (N) below each lane. (C) Soyasaponin Bb increased hZIP4 expression in AsPC1 cells. Membrane proteins (20  $\mu$ g) prepared from AsPC1 cells cultured in normal medium (N), zinc-deficient medium (CX), or in the presence of 0.01% soyasaponin Bb were immunoblotted. The ratio was quantified by densitometric analysis after normalizing the hZIP4 band intensity to that of GRP78, and is shown relative to that of AsPC1 cells cultured in normal medium (N) below each lane. (D) Soyasaponin Bb enhanced the cell surface abundance of hZIP4 in AsPC1 cells. AsPC1 cells cultured as in (C) were subjected to cell surface biotinylation assays, and the ratio was quantified and shown as described in (B). In (B and D), Tubulin and ConA were used as loading controls for input and biotinylation, respectively. (E) Soyasaponin Bb blocked hZIP4 degradation in AsPC1 cells. AsPC1 cells were cultured in zinc-deficient medium (CX) for 24 h, then cultured in the presence of 20  $\mu$ M ZnSO<sub>4</sub> (Zn) with or without 0.1% soyasaponin Bb (Bb) for 8 h. Membrane proteins were prepared and subjected to immunoblot analysis. The ratio was quantified by densitometric analysis as in (C). GRP78 is shown as a loading control in (C and E). All panels show the representative results of triplicate experiments.

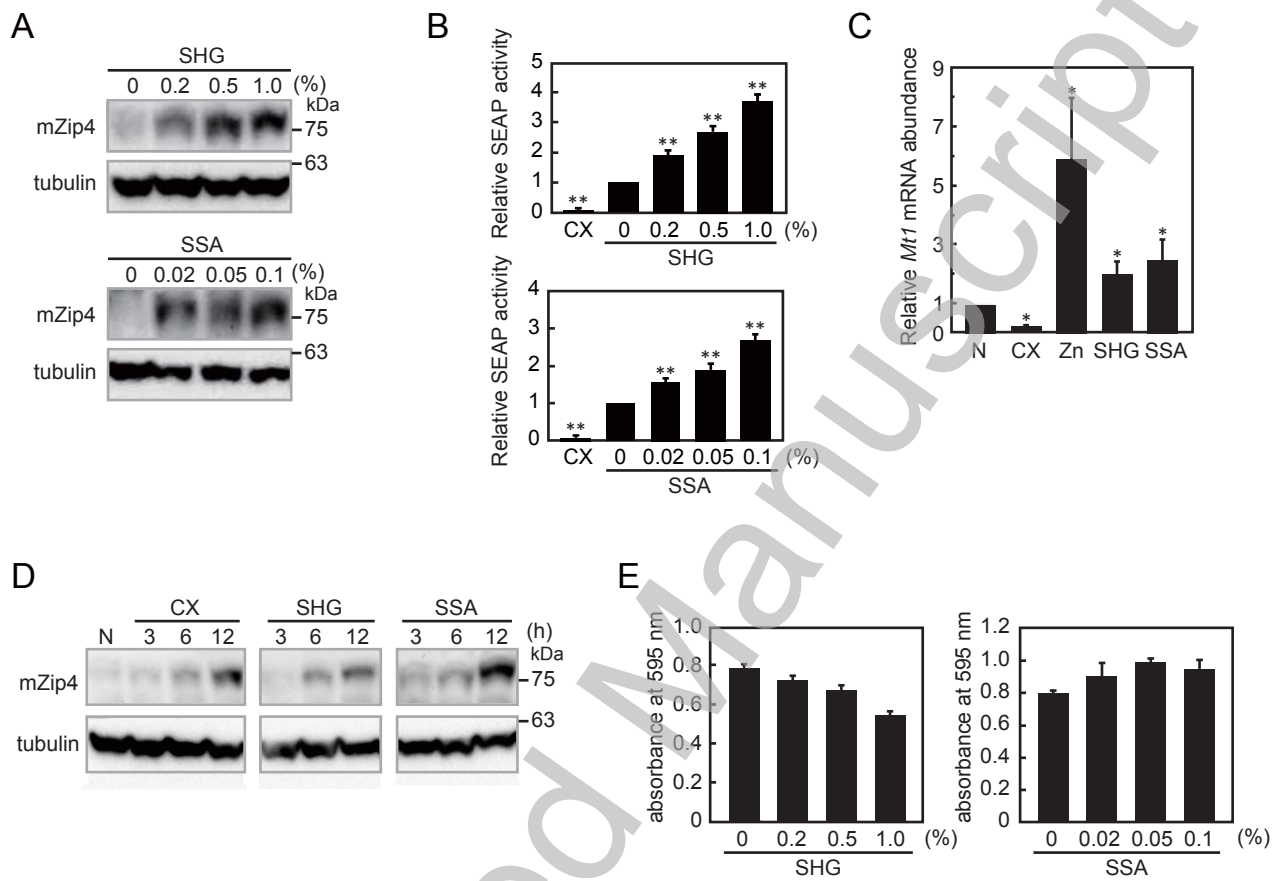


Fig. 1  
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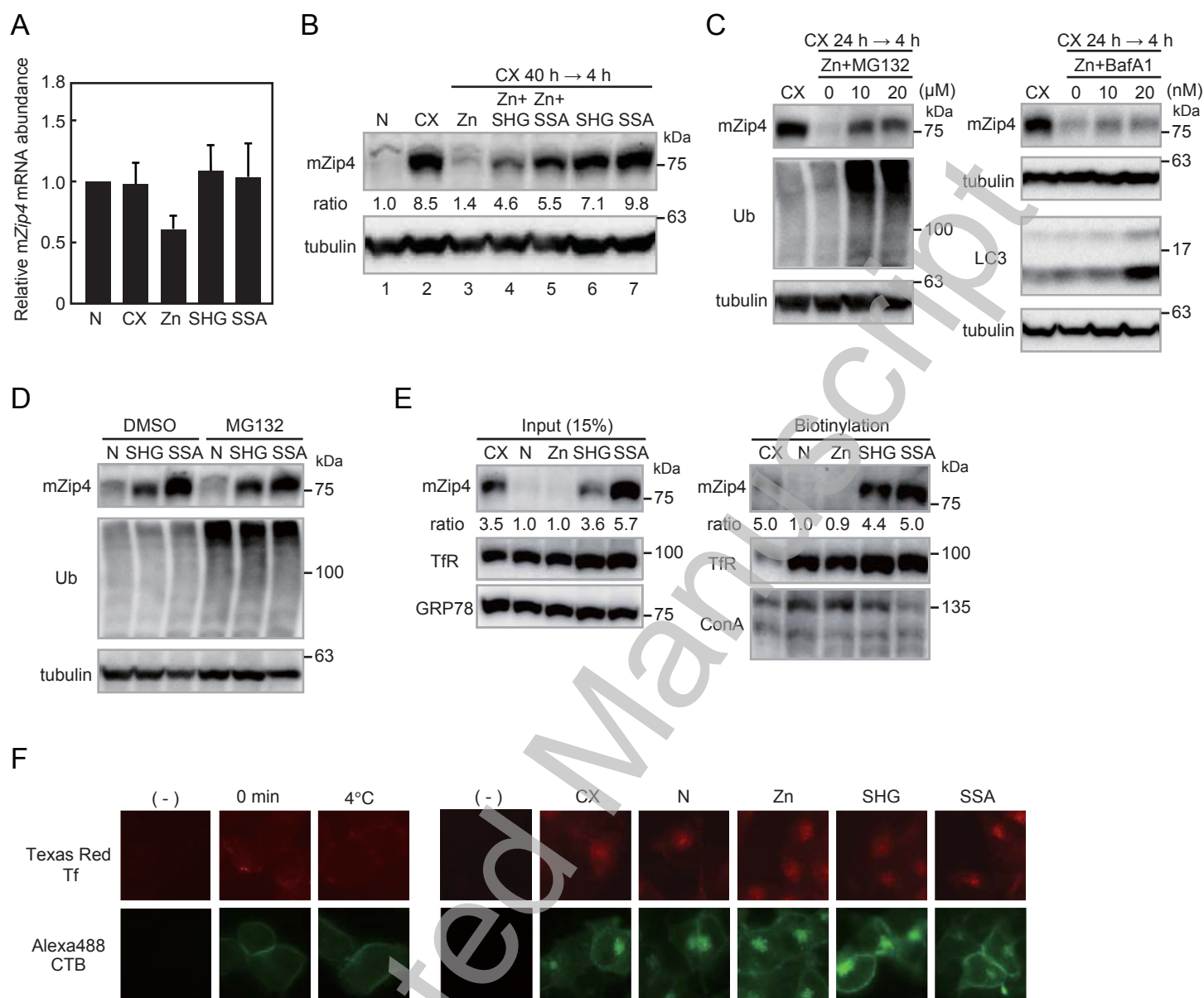


Fig.2  
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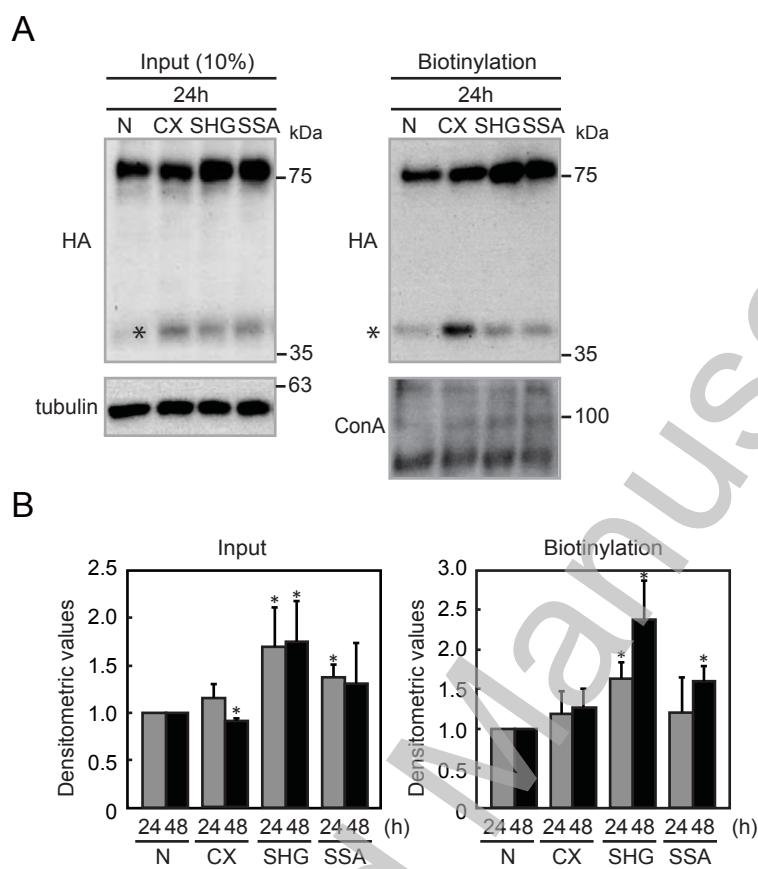


Fig. 3  
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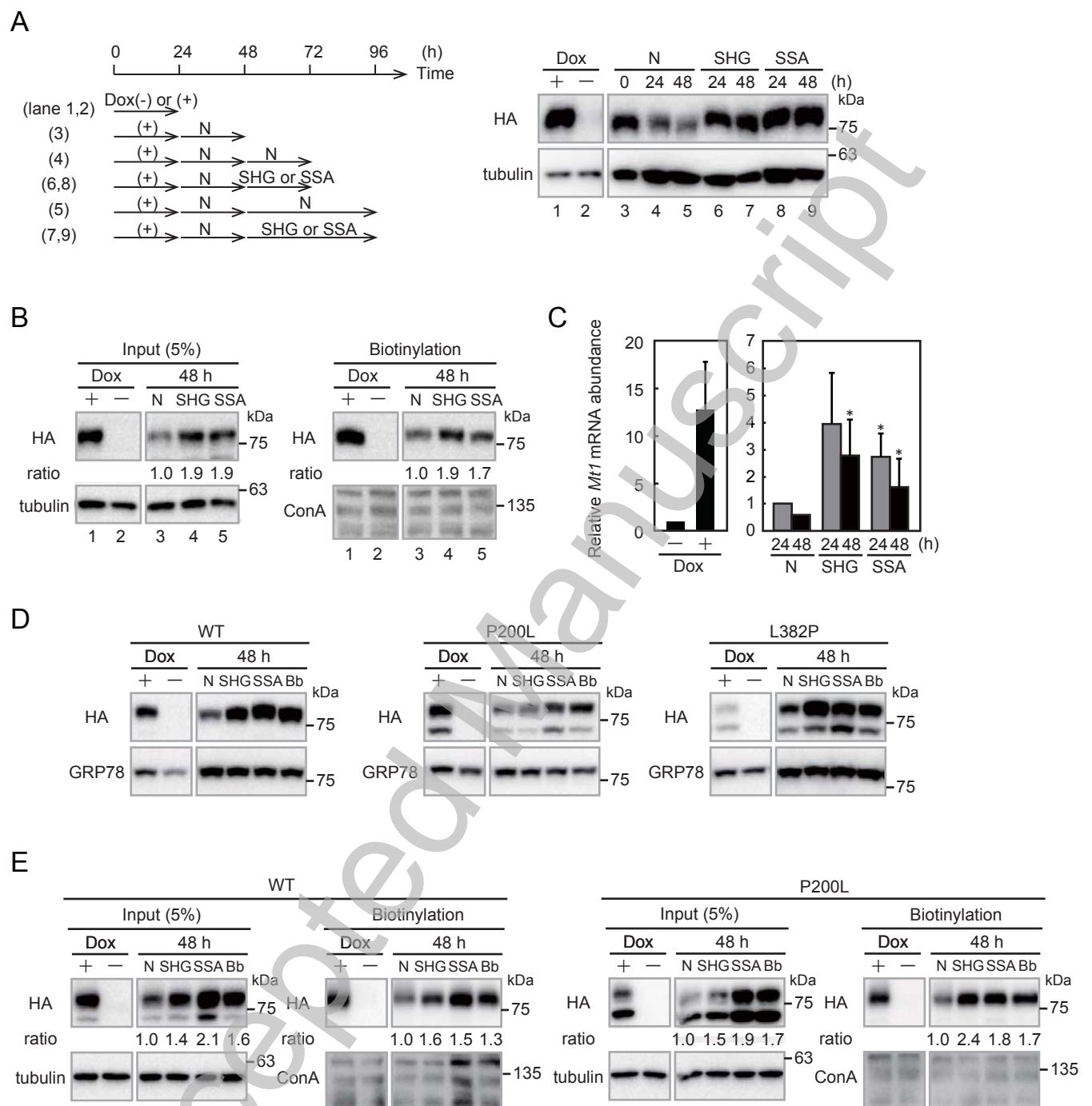


Fig. 4  
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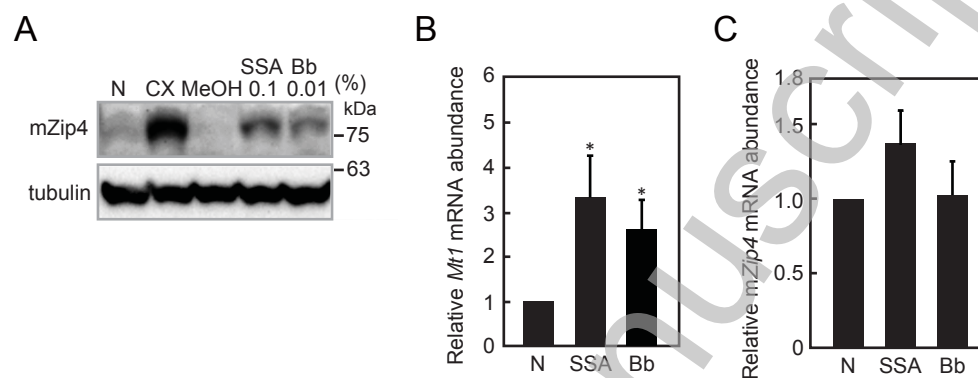


Fig. 5  
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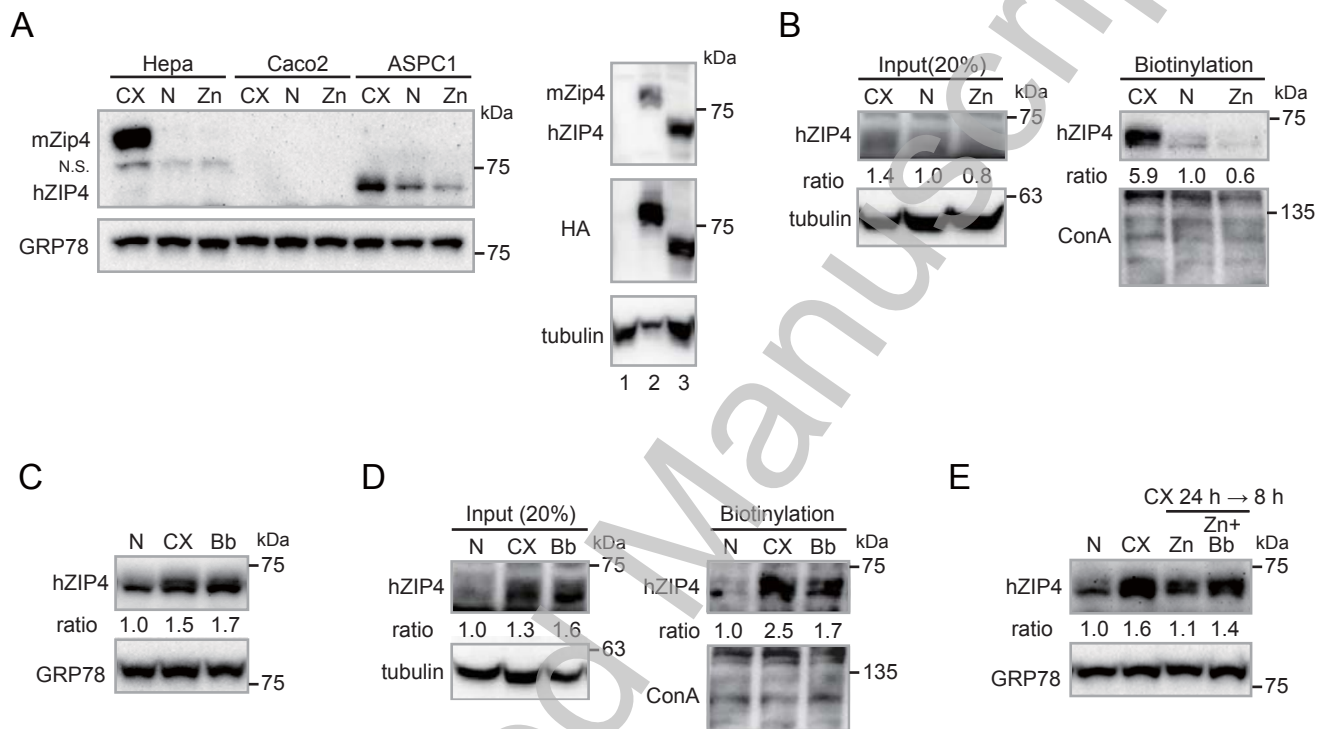


Fig. 6  
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